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THE ROLE OF CALCIUM IN THE RECEPTOR MEDIATED CONTROL OF POTASSIUM PERMEABILITY IN THE RAT LACRIMAL GLAND

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SUMMARY

1. In the presence of extracellular Ca, adrenaline stimulated a large increase in the rate of K (^{86}Rb) release from rat lacrimal slices, followed by a lower, more sustained rate.

2. In the absence of extracellular Ca, adrenaline elicited only a transient release of ^{86}Rb.

3. The artificial introduction of Ca into the cytosol by the ionophore A-23187 could also initiate the release of ^{86}Rb.

4. In a zero-Ca medium, if either adrenaline or carbachol produced a transient release of ^{86}Rb, the tissue could not respond to the other agonist with a transient release unless Ca was momentarily reintroduced to the medium.

5. If Ca was present in a limiting concentration, the Ca-dependent rate of ^{86}Rb release elicited from a lacrimal slice exposed simultaneously to carbachol and adrenaline was not significantly different from the release seen with carbachol alone.

6. It is concluded that the agonist-induced release of K from the lacrimal gland consists of both a Ca-independent phase which is initiated by the release of a limited pool of Ca, and a Ca-dependent phase which is mediated by the influx of extracellular Ca.

7. It is also concluded that both α-adrenergic and muscarinic receptor occupation activate a common, post-receptor mechanism which may be responsible for both phases of K release.

INTRODUCTION

Davson (1941) showed that erythrocytes lose potassium when F^- ions are present in the incubation medium and a later study (Gárdos, 1958) demonstrated that this loss was dependent on extracellular Ca. Subsequently, observations by Romero & Whittam (1971) and Lew (1971) indicated that Ca exerts its effect on potassium permeability through actions on the inside surface of the erythrocyte membrane. These findings have been extended to excitable (Meech, 1976) and non-excitable (Selinger, Eimerl & Schramm, 1974; Putney, 1977) tissues where an increase in cytoplasmic Ca concentration is thought to increase the membrane permeability to K.

We have shown (Putney, Parod & Marier, 1977; Parod & Putney, 1978) that the stimulation of muscarinic and α-adrenergic receptors in the rat lacrimal gland is associated with the release of K. The purpose of this investigation was to examine the possible role of Ca in the receptor mediated increase in K permeability.

METHODS

The methods for the preparation of gland slices and the measurement of K efflux with ^{86}Rb have been described previously (Parod & Putney, 1978). Gland slices were incubated in a Ringer solution of the following composition (mM): NaCl, 120; KCl, 6·2; $CaCl_2$, 3·1; $MgCl_2$, 1·6; Tris-(hydroxymethyl)aminomethane, 20; dextrose, 10. The solution was buffered with HCl to a pH of 7·40 at 37 °C and continuously gassed with 100% O_2. The slices were exposed to tracer amounts of ^{86}Rb (5–10 μc/ml.) in a normal medium for 30 min and then transferred through a series of small volumes of non-radioactive medium for 2 min each up to 50 min. The radioactivity in each incubation volume and the residual counts in the tissue after the experimental protocol were assayed with a Beckman 300 gamma counter. From these data the apparent first-order rate coefficients (percent per minute) for efflux were calculated. In some experiments, a 'zero-Ca medium' was utilized. In these experiments, the medium contained no added Ca and 10^{-4} M-(ethylenebis(oxyethylenenitrilo))tetraacetic acid (EGTA). Agonists were employed in concentrations sufficient to produce maximum responses of ^{86}Rb release; specifically, carbachol, 10^{-5} M and adrenaline, 10^{-5} M (Parod & Putney, 1978). In all of the experiments in this study, whenever an agonist, antagonist or ion was added to the solution during efflux, the substance remained until the end of the experiment.

The ^{86}Rb was purchased from New England Nuclear Corp., Boston, Mass. and had a specific activity of 400–800 c/mole. EGTA was obtained from Eastman Kodak Co., Rochester, N.Y. Carbachol, atropine, l-adrenaline and dimethyl sulphoxide (DMSO) were purchased from Sigma Chemical Co., St Louis, Mo. Divalent cationophore, A-23187, was kindly supplied by Eli Lilly Co., Indianapolis, Ind. Phentolamine HCl was a gift from the Ciba-Geigy Corp., Summit, N.J.

Summarized data are represented by the arithmetic mean, and dispersions are not shown as they generally averaged less than 10% of the mean. Statistical comparisons were made using a two-way analysis of variance with the critical probability for rejection of the null hypothesis being 0·05.

RESULTS

Adrenaline induces a significant increase in membrane permeability to K, as determined with the ^{86}Rb technique (Fig. 1). The kinetics of release appear biphasic in that initially there is a large increase in the rate of release which is followed by a lower, more sustained phase. Both phases are blocked by phentolamine. Although there is a small but significant increase in ^{86}Rb release in the presence of phentolamine, the increase appears to be a response of an unknown mechanism and not α-receptor mediated (Parod & Putney, 1978). Again, while phentolamine blocks both phases, omission of Ca from the incubation media blocks only the sustained phase of ^{86}Rb release (Fig. 1). This response is reversible in that the reintroduction of Ca in the presence of adrenaline increases the release of ^{86}Rb. However, if adrenaline is not present, the reintroduction of Ca actually decreases the rate of ^{86}Rb release.

Evidence which further supports a role for Ca in ^{86}Rb release was obtained from experiments with the divalent ionophore A-23187. Ionophore, dissolved in DMSO, was added to a zero-Ca medium to a final concentration of 20 μM. Control slices were exposed to the same solvent concentration (1%) but without ionophore. Fig. 2 shows the tissue response when $CaCl_2$ was added to both media to a final concentration of 3·3 mM (3·2 mM ionized, since 10^{-4} M-EGTA was still present). Fig. 2*A* demonstrates that the introduction of Ca to tissue exposed to either DMSO or DMSO + ionophore will elicit the release of ^{86}Rb. However, if the same experiment (Fig. 2*B*) is conducted in the presence of atropine and phentolamine, the addition of Ca will cause a response only when the ionophore is present.

We have shown previously (Putney *et al.* 1977*a*) that, like adrenaline, the release

of ^{86}Rb due to carbachol is also biphasic in nature. Both phases are blocked by atropine. The initial peak ^{86}Rb release is independent of extracellular Ca, while only the lower, more sustained rate of release requires the presence of Ca in the bathing media. Thus the effect of Ca on the release of ^{86}Rb by carbachol is qualitatively and temporarily similar to the effect of Ca on the adrenaline response (Fig. 1). With these similarities in mind, it was of interest to examine the nature of the transient phase of K release.

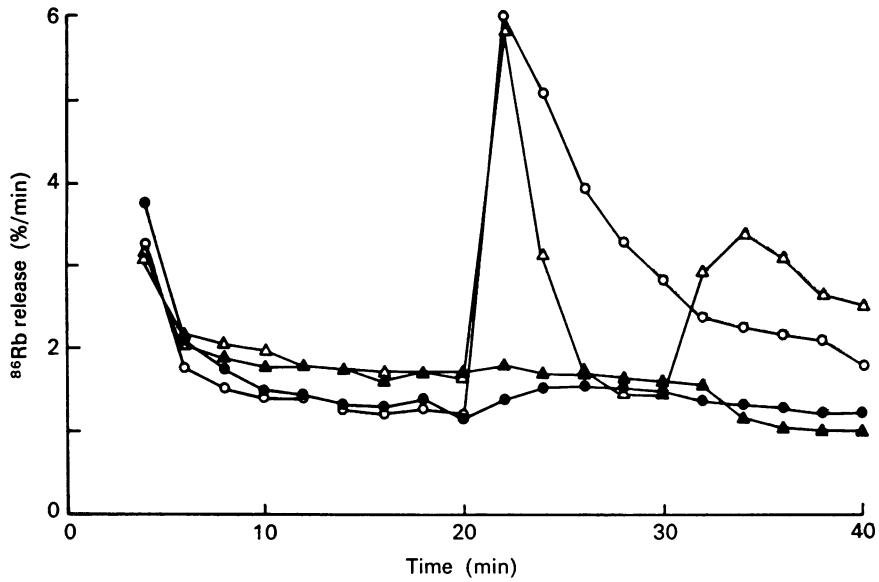

Fig. 1. Release of ^{86}Rb due to adrenaline. ○—○, 10^{-5} M-adrenaline 20–40 min; ●—●, 10^{-4} M-phentolamine 14–40 min, 10^{-5} M-adrenaline 20–40 min; △—△, no added Ca + 10^{-4} M-EGTA 0–40 min, 10^{-5} M-adrenaline 20–40 min, $3{\cdot}3 \times 10^{-3}$ M-Ca 30–40 min; ▲—▲, no added Ca + 10^{-4} M-EGTA 0–40 min, $3{\cdot}3 \times 10^{-3}$ M-Ca 30–40 min. Each point is the mean of three separate experiments; i.e. four slices from each of three rats were subjected to the four protocols. The S.E. of the mean for all data points averaged less than 10% of the mean.

Fig. 3 shows that the addition of carbachol to tissue slices incubated in zero-Ca media caused a transient release of ^{86}Rb and inactivated the transient release of ^{86}Rb due to the subsequent administration of adrenaline. However, the transient response to adrenaline at a later time could be restored if Ca was momentarily added to the incubation media (i.e. added for two minutes and then removed by excess EGTA). In the converse experiment (i.e. adrenaline added first, followed by carbachol, data not shown) the same results were obtained. In Fig. 3 the addition of Ca at first appears to give a response similar to the Ca-independent phase shown in Fig. 1; however, if instead of adding antagonist the incubation was continued for a longer period of time, the response would be identical to the lower, Ca-dependent phase of ^{86}Rb release as seen in Fig. 1 for adrenaline and as shown by Putney *et al.* (1977*a*) for carbachol.

The terms 'Ca-independent' and 'Ca-dependent' are used only to describe the original observation that the release of ^{86}Rb due to receptor activation consists of two phases, one independent

of and one dependent on the presence of Ca in the extracellular space. These phases are not intended to imply that only one phase is mediated by Ca, indeed, as this manuscript purports, both phases of release may ultimately be initiated by a rise in intracellular Ca.

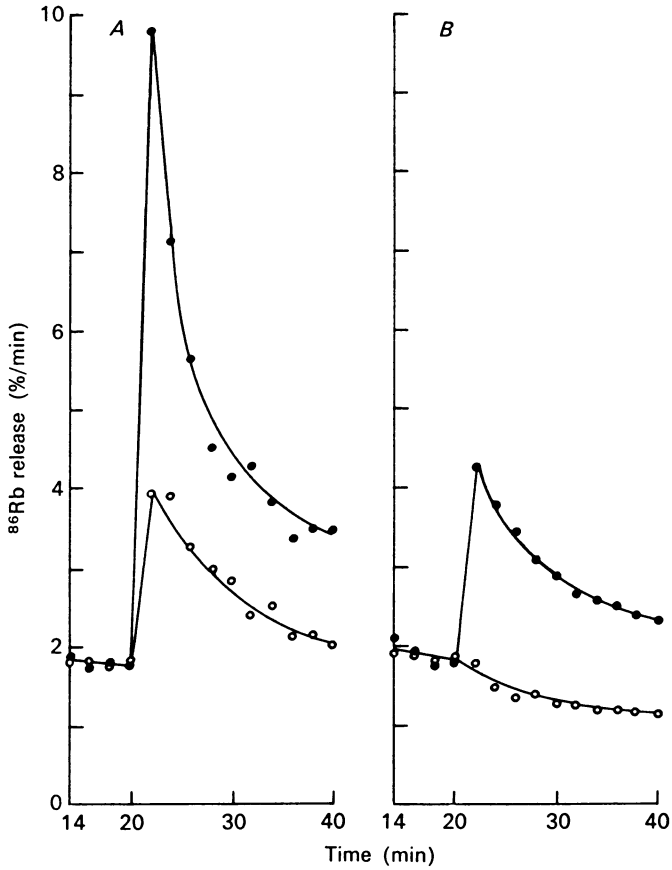

Fig. 2. Release of ^{86}Rb due to the addition of Ca in the presence of ionophore A-23187 + dimethyl sulphoxide (DMSO) or DMSO alone. The following concentrations were employed: A-23187, 2×10^{-5} M; DMSO, 1%; Ca, $3{\cdot}3 \times 10^{-3}$ M; atropine, 10^{-6} M; phentolamine, 10^{-6} M. The incubation media contained no added Ca + 10^{-4} M-EGTA. *A*: ●—●, A-23187 + DMSO 10–40 min, Ca 20–40 min; ○—○, DMSO 10–40 min, Ca 20–40 min. *B*: ●—●, A-23187 + DMSO 10–40 min, Ca, atropine and phentolamine 20–40 min; ○—○, DMSO 10–40 min, Ca, atropine and phentolamine 20–40 min. Each point is the mean of four paired experiments. The values for S.E. averaged less than 10% of the means.

It might be argued that the tissue failed to respond to the second agonist because of prolonged incubation in zero-Ca or that the first drug and its antagonist prevented receptor activation by the second agonist in some non-specific manner. It seems unlikely that the addition of Ca for 2 min could reverse either of these effects, but in order to examine these possibilities we performed the experiments shown in Fig. 4. After 40 min in zero-Ca medium, the tissue was still able to respond to adrenaline. Also, if the response to carbachol was blocked by the prior administration of atropine,

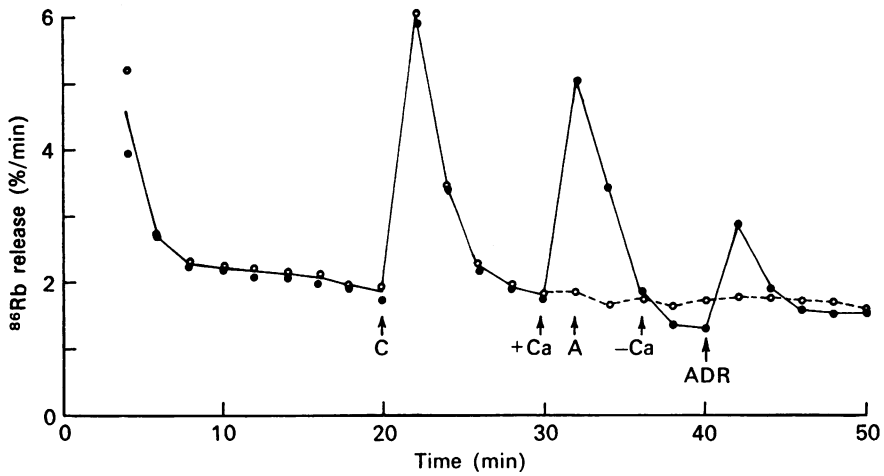

Fig. 3. Release of ^{86}Rb due to the series addition of carbachol and adrenaline. Lacrimal slices were loaded with ^{86}Rb for 30 min and then sequentially carried through a series of 2 min incubations in a zero-Ca solution for 50 min. Agents were added at the times indicated by the arrows and were always present until the end of the experiment. Concentrations employed were: carbachol, 10^{-5} M; adrenaline, 10^{-5} M; atropine, 10^{-6} M; Ca, 10^{-3} M; EGTA, 3×10^{-3} M. ○—○, C = carbachol; ADR = adrenaline. ●—●, C = carbachol; +Ca = Ca; A = atropine; −Ca = EGTA; ADR = adrenaline. Each point is the mean of three paired experiments. The values for S.E. averaged less than 10% of the means.

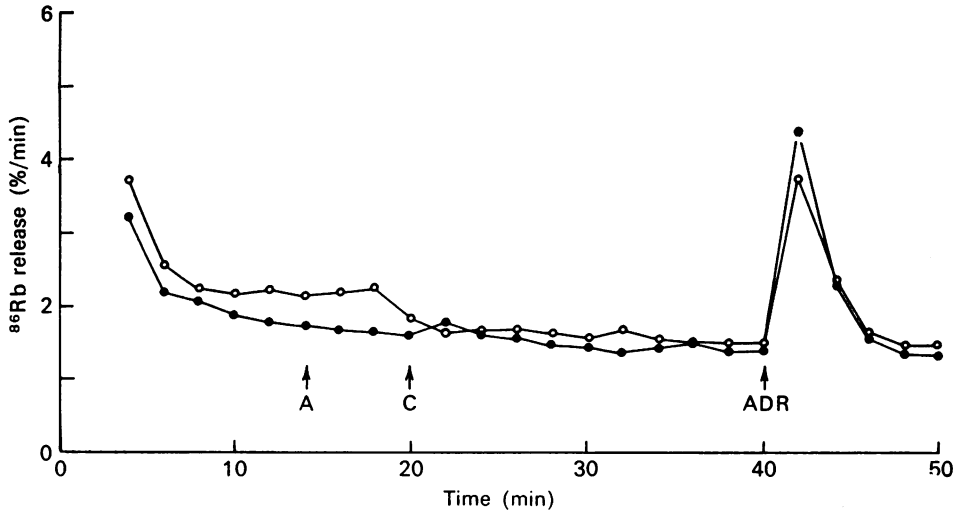

Fig. 4. Release of ^{86}Rb due to the series addition of carbachol and adrenaline. The protocol was similar to that for Fig. 3. Agents were added at the times indicated and were always present until the end of the experiment. Concentrations employed were: carbachol, 10^{-5} M; adrenaline, 10^{-5} M; atropine, 10^{-6} M. ○—○, ADR = adrenaline. ●—●, A = atropine; C = carbachol; ADR = adrenaline. Each point is the mean of three paired experiments. The values for S.E. averaged less than 10% of the means.

adrenaline gave a normal response at 40 min. A two-way analysis of variance showed that the responses to adrenaline in the two different experimental protocols did not differ significantly. In the converse experiment (i.e. carbachol added at 40 min with or without the prior administration of phentolamine and adrenaline) a similar pattern of responses was seen (data not shown).

Fig. 3 previously indicated that the transient presence of extracellular Ca is required to obtain a response to the second agonist. We wondered if this reactivation phenomenon was dependent on the reintroduction of Ca during receptor activation.

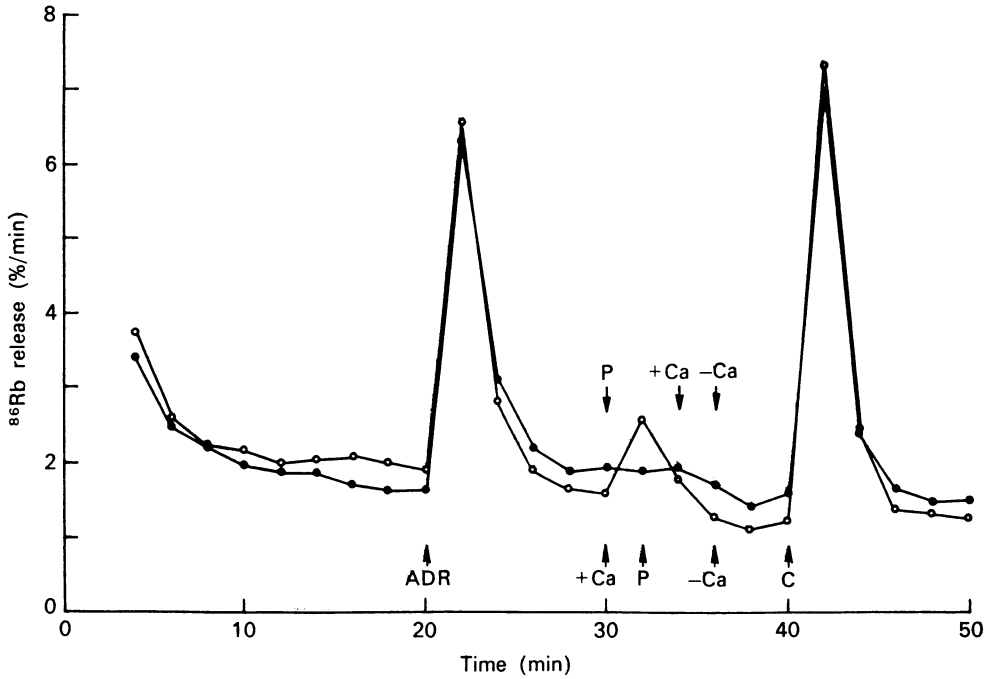

Fig. 5. Release of ^{86}Rb due to the series addition of adrenaline and carbachol. The protocol was similar to that for Fig. 3. Agents were added at the times indicated and were always present until the end of the experiment. Concentrations employed were: carbachol, 10^{-5} M; adrenaline, 10^{-5} M; phentolamine, 10^{-6} M; Ca, 10^{-3} M; EGTA, 3×10^{-3} M. ○—○, ADR = adrenaline; +Ca = Ca; (P) = phentolamine; −Ca = EGTA; C = carbachol. ●—●, ADR = adrenaline; P = phentolamine; +Ca = Ca; −Ca = EGTA; C = carbachol. Each point is the mean of three paired experiments. The values for S.E. averaged less than 10% of the means.

To answer this question we performed the experiments shown in Fig. 5. It made no difference whether Ca was added 2 min before phentolamine (i.e. receptor in active state) or 4 min after phentolamine (i.e. receptor inactivated); there was no statistical difference in the release of ^{86}Rb upon introduction of the second agonist (carbachol). Again in the converse experiment (i.e. carbachol added at 20 min, followed by atropine before or after the reintroduction of Ca, followed by adrenaline at 40 min) there was no statistical difference in the response to the second agonist (data not shown).

Since the results obtained thus far suggest a common mechanism with respect to

the transient phase of ^{86}Rb release, we further investigated the possibility that a similar situation might occur for the sustained phase. These experiments are summarized in Figs. 6 and 7.

Fig. 6 indicates that in the presence of carbachol, the reintroduction of Ca into a zero-Ca medium caused an increase in the rate of ^{86}Rb release and the magnitude of this increase was related to the concentration of Ca added. Since both carbachol and adrenaline were capable of eliciting a release of ^{86}Rb which was dependent on extracellular Ca, we wondered if the individual Ca-dependent phases of release would summate if both agonists were added at the same time.

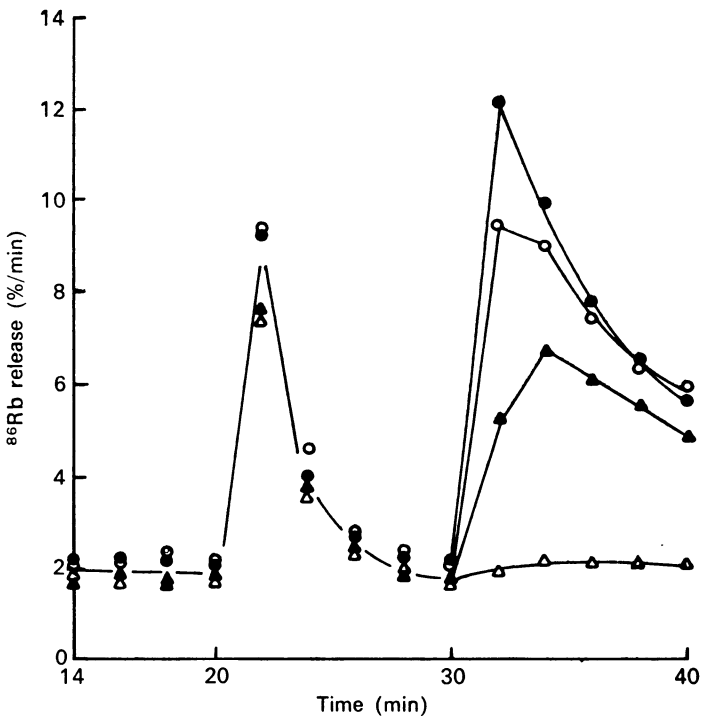

Fig. 6. Release of ^{86}Rb due to carbachol at various Ca concentrations. The protocol was similar to that for Fig. 3. In all cases incubations were performed with no added Ca + 10^{-4} M-EGTA 0–40 min and 10^{-5} M carbachol 20–40 min. △—△, 3×10^{-4} M-Ca 30–40 min; ▲—▲, 10^{-3} M-Ca 30–40 min; ○—○, 3×10^{-3} M-Ca 30–40 min; ●—●. 10^{-2} M-Ca 30–40 min. Each point is the mean of three paired experiments. The values for S.E. averaged less than 15% of the means.

Fig. 7 shows that, in fact, this does not occur. If Ca is present in a limiting concentration, the tissue's response to the simultaneous administration of maximal concentrations of carbachol and adrenaline was not significantly different than its response to a maximal concentration of carbachol alone.

DISCUSSION

Fig. 1 shows that the release of ^{86}Rb in response to the addition of adrenaline is biphasic in nature, consisting of a large increase in the rate of release followed by a lower, more sustained rate. If adrenaline is added to a zero-Ca medium, only the initial release of ^{86}Rb is seen and the rate of release quickly returns to basal levels in 6–8 min. If Ca is now returned to the medium, an increase in ^{86}Rb release is initiated which is similar in magnitude to the sustained release seen in the control response to adrenaline. Although adrenaline is slightly less efficacious, the response

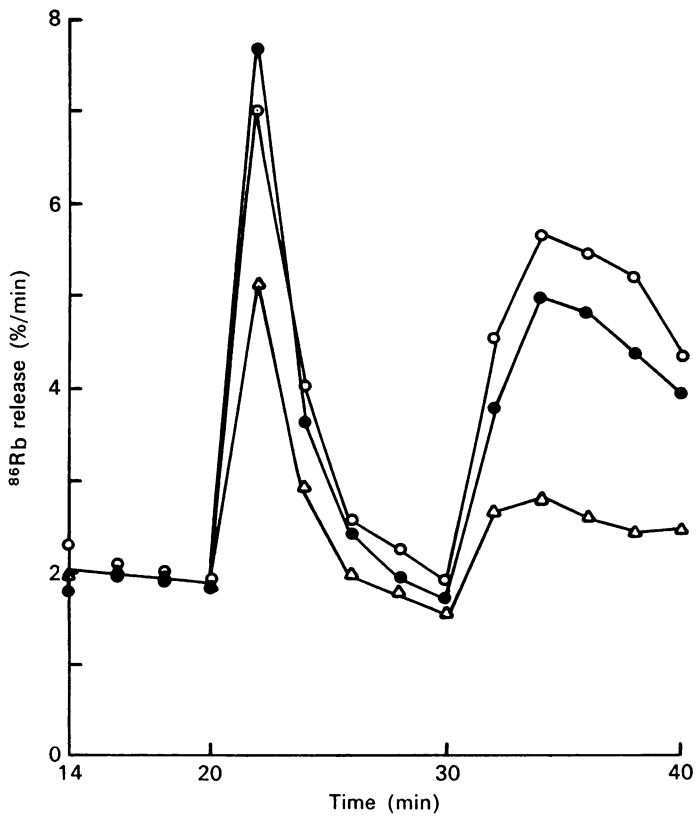

Fig. 7. Release of ^{86}Rb. The protocol was similar to that for Fig. 3. In all cases incubations were performed with no added Ca + 10^{-4} M-EGTA 0–40 min and 10^{-3} M-Ca 30–40 min. ○—○, 10^{-5} M-carbachol 20–40 min ($n = 3$); ●—●, 10^{-5} M-carbachol + 10^{-5} M-adrenaline 20–40 min ($n = 6$); △—△, 10^{-5} M-adrenaline 20–40 min ($n = 3$). The values for S.E. averaged less than 10% of the means.

of the lacrimal gland to this agonist, both in the presence and absence of extracellular Ca, is similar to the response elicited by carbachol (Putney *et al.* 1977*a*). Thus, it appears that muscarinic and α-adrenergic stimulation serve to release K by both a Ca-independent and a Ca-dependent mechanism.

With regard to the Ca-dependent phase of K release, it has been previously noted (Douglas & Rubin, 1963) that the addition of Ca after perfusion with a Ca-free

solution is in itself sufficient to release catecholamines from the adrenal gland. Thus, it might be argued that the Ca-dependent phase of K release may be a similar phenomenon. Fig. 1 demonstrates that this is not the case as the reintroduction of Ca to slices incubated in zero-Ca actually decreases ^{86}Rb efflux. Observations in other excitable and non-excitable tissues (Putney, 1977) indicate that the omission of extracellular Ca generally enhances membrane permeability to various ions. Our study with the lacrimal gland indicates that this tissue is similarly affected. While the absence of extracellular Ca slightly but significantly increases the rate of ^{86}Rb release, the rate of release rapidly reverts to control levels when Ca is returned to the incubation media. Thus it would appear that the Ca-dependent phase of K release is a receptor related event. However, at least two possibilities can be envisioned, (1) Ca is required in some way for receptor activation or (2) it is involved at some subsequent step.

The artificial introduction of Ca into the cell via the divalent cationophore A-23187 (Fig. 2) provides evidence for the second supposition. Ca added to media containing DMSO or DMSO + ionophore causes the release of ^{86}Rb. However, much of this effect may be due to the release of endogenous stores of neurotransmitter as Fig. 2*B* suggests. When the muscarinic and α-adrenergic receptors are blocked by atropine and phentolamine respectively, the increase in ^{86}Rb release due to the ionophore-mediated introduction of Ca into the cytoplasm is unmasked. Thus the lacrimal gland appears similar to the parotid gland where, in the presence of ionophore A-23187, Ca has also been reported to cause K release (Selinger *et al.* 1974). In addition to these observations with ionophore, the influx of ^{45}Ca due to muscarinic and α-adrenergic receptor activation has been reported in a number of tissues (in rabbit aortic smooth muscle by noradrenaline, Deth & van Breemen, 1974; in isolated rat pancreas cells by carbachol, Kondo & Schulz, 1976; in rat lacrimal gland by carbachol, Keryer &Rossignol, 1976; in rat parotid slices by muscarinic and α-adrenergic stimulation, Putney, 1976 and Putney, Weiss, Leslie & Marier, 1977; in isolated rat submandibular and parotid cells by muscarinic and α-adrenergic stimulation, Koelz, Kondo, Blum & Schulz, 1977). These data are not inconsistent with the idea that in these tissues and in the lacrimal gland, muscarinic and α-adrenergic stimulation serve to activate a population of Ca influx sites which in turn elicit appropriate cellular responses by increasing the cytoplasmic Ca concentration.

The question now arises: are there two distinct populations of influx sites, one controlled by carbachol and one by adrenaline, or is there a single population responsive to both agonists. Fig. 7 argues for the latter supposition. If muscarinic and α-adrenergic stimulation each activated a separate Ca influx site, one would predict that the Ca-dependent release initiated by the addition of carbachol would be increased by the co-administration of adrenaline, as the second agonist would serve to add more Ca to the cytosol. However, Fig. 7 shows that when the ability of the tissue to release ^{86}Rb is limited by the extracellular Ca concentration, the Ca-dependent response elicited by carbachol was not enhanced with addition of adrenaline. Thus both agonists seem to not only cause an influx of Ca but also to activate the same influx sites.

While it appears that the influx of Ca mediates the sustained release of K this does not explain the nature of the transient K release seen in the absence of extracellular

Ca. Fig. 3 shows that a tissue slice incubated in a zero-Ca medium will respond to the addition of agonist with a transient release of ^{86}Rb. However, if a second agonist known to cause K release is added subsequently, the tissue is unable to respond. A similar observation was also noted in aortic smooth muscle by Deth & van Breemen (1977). These authors found that in the absence of extracellular Ca, noradrenaline produced a transient contraction and if noradrenaline was then removed and subsequently reintroduced, the tissue failed to respond with another transient contraction. Since a rise in intracellular Ca is involved in the release of ^{86}Rb from the lacrimal gland (Fig. 2) and in contraction of smooth muscle, these data can be interpreted to represent the agonist-induced release of a limited pool of cellular Ca. Thus, if no extracellular Ca is present to supplement the agonist-induced response, the release of a limited pool of Ca would explain the transient release of ^{86}Rb and contraction seen with these tissues. The existence of this Ca pool is supported by our observation that briefly exposing the tissue to extracellular Ca will restore the transient release of ^{86}Rb upon the addition of a second agonist (Fig. 3). Further support comes from Deth & van Breemen (1977) who noted that, in a zero-Ca medium, α-adrenergic stimulation of aortic rings previously loaded with ^{45}Ca produces a transient increase in nuclide efflux which also fails upon reintroduction of agonist at a later time.

Fig. 3 and the converse experiment described in *Results* also indicate that the limited pool of intracellular Ca is responsive to both muscarinic or α-adrenergic stimulation (i.e. once released through the action of one agonist, it cannot respond to the other). This same phenomenon was noted by Deth & van Breemen (1974) who observed that noradrenaline, angiotensin and histamine could all mobilize the same Ca fraction. Our findings suggest not only that muscarinic and α-adrenergic receptor activation, through some common mechanism, release Ca from a cellular store, but also that these receptors must be located on the same cell in order to do so. Arguments suggesting that this cell is in fact the lacrimal gland acinar cell have been provided previously (Putney *et al.* 1977*a*; Parod & Putney, 1978).

Data on the cellular nature of this Ca pool are presently lacking. However, Fig. 5 indicates that the addition of Ca to the extracellular space for 2 min is sufficient to reload this pool completely; it makes no difference whether the Ca influx sites are in an active or inactive state. Exactly what this implies about the kinetic nature of this Ca pool has yet to be ascertained.

Since this Ca pool in the lacrimal gland is responsive to both carbachol and adrenaline, and since the Ca influx sites in these tissues also respond to both agonists, this may imply that both the Ca-independent and Ca-dependent phases of K release are mediated by the same mechanism which is in turn activated by both muscarinic and α-adrenergic stimulation. Evidence which supports this contention comes from our earlier observation (Parod & Putney, 1978) that the dose–response curves of the Ca-independent and Ca-dependent phases of ^{86}Rb release to both agonists can be practically superimposed if the responses are scaled to the same maximum. While this evidence is not conclusive and other possibilities can be envisioned, we have not found any data to date which would suggest that both phases are not initiated through activation of the same receptor.

In conclusion we have shown that the agonist induced release of ^{86}Rb can be divided into a Ca-dependent and a Ca-independent phase. The Ca-dependent release

of K is mediated by the influx of extracellular Ca, while the Ca-independent phase of K release is initiated by the release of a limited pool of bound Ca. Both phases appear to be activated by either muscarinic or α-adrenergic receptor-mediated stimulation of some common mechanism located in or on the acinar cell membrane.

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